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The Novel Parainfluenza Virus Hemagglutinin-Neuraminidase Inhibitor BCX 2798 Prevents Lethal Synergism between a Paramyxovirus and *Streptococcus pneumoniae*

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An association exists between respiratory viruses and bacterial infections. Prevention or treatment of the preceding viral infection is a logical goal for reducing this important cause of morbidity and mortality. The ability of the novel, selective parainfluenza virus hemagglutinin-neuraminidase inhibitor BCX 2798 to prevent the synergism between a paramyxovirus and *Streptococcus pneumoniae* was examined in this study. A model of secondary bacterial pneumonia after infection with a recombinant Sendai virus whose hemagglutinin-neuraminidase gene was replaced with that of human parainfluenza virus type 1 [rSV(hHN)] was established in mice. Challenge of mice with a sublethal dose of *S. pneumoniae* 7 days after a sublethal infection with rSV(hHN) (synergistic group) caused 100% mortality. Bacterial infection preceding viral infection had no effect on survival. The mean bacterial titers in the synergistic group were significantly higher than in mice infected with bacteria only. The virus titers were similar in mice infected with rSV(hHN) alone and in dually infected mice. Intranasal administration of BCX 2798 at 10 mg/kg per day to the synergistic group of mice starting 4 h before virus infection protected 80% of animals from death. This effect was accompanied by a significant reduction in lung viral and bacterial titers. Treatment of mice 24 h after the rSV(hHN) infection showed no protection against synergistic lethality. Together, our results indicate that parainfluenza viruses can prime for secondary bacterial infections. Prophylaxis of parainfluenza virus infections with antivirals might be an effective strategy for prevention of secondary bacterial complications in humans.

It is well known that respiratory viruses can predispose for bacterial disease. This association came into particular focus as the influenza pandemic of 1918 took its toll of some 40 to 50 million lives (39), many of them due to secondary bacterial pneumonia (34, 51). This event was the genesis of investigations into the epidemiology and pathology of viral-bacterial infections revealing other associations between pathogens that cause infections of the upper and lower respiratory tract. Since that time, studies with humans have shown that respiratory viruses can enhance bacterial colonization of the nasopharynx (4, 13, 44, 49) and facilitate bacterial invasion and spread to the middle ear (21, 48), the sinuses (12, 17), and the lungs (25, 28). This association is most evident for influenza virus because of the striking impact on mortality from secondary bacterial pneumonia (43). However, members of the *Paramyxoviridae* family, particularly respiratory syncytial virus (RSV) and human parainfluenza viruses (hPIVs), have also been implicated in the pathogenesis of bacterial otitis media (5, 20, 21, 41), sinusitis (18), and pneumonia (25, 28). Other respiratory viruses, including rhinoviruses and adenoviruses, might also be involved in this interaction, although the relative contributions of each virus or each virus-bacterium pair are only now being unraveled.

Interventions aimed at prevention or treatment of influenza virus or RSV infections have led to a reduction in secondary bacterial complications. Vaccination of children against influenza A virus was not only protective against influenza virus-induced illness but also decreased the incidence of bacterial otitis media (2, 6, 18). In vaccinated adults, a similar protective effect can be seen against secondary bacterial pneumonia (14, 36, 37, 38). Although no vaccines are currently licensed for use against RSV or hPIVs, passive immunization with a high dose of polyclonal RSV antibody preparation has been demonstrated to prevent otitis media (42). In cases in which prevention of preceding viral infection cannot be accomplished or has been inadequate, the use of antiviral drugs might be an appropriate strategy to prevent later bacterial complications. Among viral respiratory illnesses, effective inhibitors are licensed only for influenza viruses at present. Zanamivir and oseltamivir are two members of a novel class of potent and selective inhibitors of the influenza virus neuraminidase (NA) that have been licensed for treatment of influenza A and B virus infections in recent years (16). Although no studies primarily designed to determine the effect of these drugs on prevention of secondary complications of influenza in humans have been published, some effects can be seen even with this caveat. Early oseltamivir treatment reduced the development of acute otitis media by 44% in influenza-infected children 1 to 12 years old (50). In adults, early oseltamivir or zanamivir treatment of influenza reduced the occurrence of secondary complications (otitis media, sinusitis, bronchitis, or pneumonia), as well as antibiotic use (33, 47). Inhibitors of the human parainfluenza virus hem-

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agglutinin-NA (HN) might be expected to have similar effects on secondary bacterial infections after infections with PIVs.

This is the first report describing the efficacy of the novel parainfluenza virus HN inhibitor BCX 2798 in the prevention of secondary *Streptococcus pneumoniae* infections in an animal model. BCX 2798 was designed and synthesized upon the available crystal structure of the HN protein of Newcastle disease virus (NDV) (9, 45). We previously reported the efficacy of this novel compound against parainfluenza viruses both in vitro and in vivo (1). BCX 2798 successfully inhibited binding and NA activities of hPIV-1, hPIV-2, and hPIV-3 viruses (with the highest level activity against the HN of hPIV-1) and was highly efficacious in prophylaxis of lethal infection in mice with a recombinant parainfluenza virus bearing the hPIV-1 HN (instead of the Sendai virus [SV] HN) on an SV background. This virus, denoted rSV(hHN), was used in a prior study (1) because the hPIVs infect experimental animals poorly, infection is usually asymptomatic, and lung pathology is minimal or undetectable (7, 29, 35, 46). In contrast, infection of mice with the recombinant virus rSV(hHN) causes severe illness and robust replication in the lungs of mice (1). In the present study we utilized this virus model to develop and characterize the synergism between PIVs and *S. pneumoniae* in mice. We hypothesized that the novel parainfluenza virus inhibitor BCX 2798 would prevent the synergistic mortality caused by rSV(hHN) and pneumococcus. The experiments demonstrate that rSV(hHN) predisposes for development of lethal pneumococcal pneumonia in mice, and the BCX 2798 compound prevents this lethal synergism in a prophylaxis treatment model.

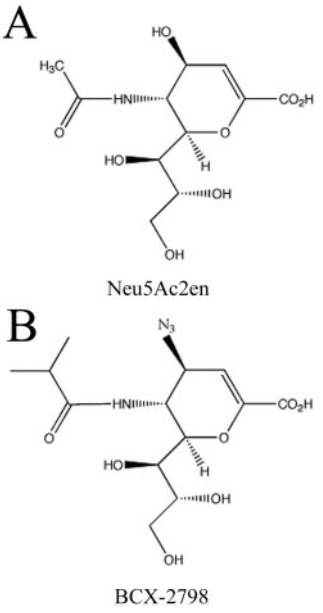

FIG. 1. Chemical structure of BCX 2798 and its parent compound. (A) Neu5Ac2en (2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid); (B) BCX 2798 (4-azido-5-isobutyrylamino-2,3-didehydro-2,3,4,5-tetradeoxy-D-glycero-D-galacto-2-nonulopyranosic acid).

MATERIALS AND METHODS

Compound. BCX 2798 (4-azido-5-isobutyrylamino-2,3-didehydro-2,3,4,5-tetradeoxy-D-glycero-D-galacto-2-nonulopyranosic acid) was synthesized by BioCryst Pharmaceuticals, Inc. (Birmingham, Ala.), through structure-based drug design based on the structure of the lead compound, Neu5Ac2en (2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid) (Fig. 1A), bound to the active site of NDV HN. Analysis of the Neu5Ac2en-HN complex suggested that a bulkier hydrophobic group could be accommodated in place of the methyl group of the acetamido moiety at C-5. BCX 2798 is the derivative of Neu5Ac2en in which the O_4 hydroxyl group has been replaced by an azido group, and the methyl group of the acetamido moiety at C-5 has been replaced by an isopropyl group (Fig. 1B). The compound was provided as lyophilized powder and stored at 4°C. BCX 2798 was solubilized in water before inoculation at a concentration appropriate to the dose to be administered.

Cell cultures. LLC-MK_2 cells were obtained from the American Type Culture Collection (Manassas, Va.) and were grown in Eagle minimal essential medium containing 5% fetal bovine serum in a humidified atmosphere of 5% CO_2. Gentamicin (BioWhittaker, Walkersville, Md.) was added at a concentration of 50 μg/ml during growth of viral stocks. The 293T (human kidney epithelial) cells (11) used for the rescue of rSV(hHN) were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum.

Infectious agents. rSV(hHN) virus, which contains the hPIV-1 HN gene instead of the HN gene of SV, was rescued by reverse genetic methods. Briefly, SV (strain E) was rescued from the full-length SV cDNA genome pSeV(+) as described previously (26). The full-length cDNA clone of SV was mutated to include an NotI site upstream and an AscI site downstream of the HN gene, creating the pSV(+)AN plasmid. These restriction sites were also added to the hHN cDNA (from hPIV-1) and used to exchange the HN gene. For the rescue of the recombinant virus, 293T cells were infected with the vaccinia virus vTF7-3, which expresses T7 RNA polymerase, and transfected with the full-length rSV(hHN) genome, as well as the NP, P, and L genes in expression vectors as described previously (3). Two days after transfection, the infected and transfected cells were subjected to three cycles of freezing and thawing and injected into the allantoic cavity of 10-day-old embryonated chicken eggs to amplify the virus. The rescued virus was plaque purified on LLC-MK_2 cells, amplified in embryonated chicken eggs, divided into aliquots, and kept frozen at −70°C. The sequence of the HN gene of egg-grown rSV(hHN) did not differ from that of wild-type hPIV-1. Infectivity of virus was determined by endpoint dilution assay in LLC-MK_2 [to determine the dose(s) of virus in 1 ml that infects 50% of cells in culture ($TCID_{50}$/ml)]. *S. pneumoniae* D39, a type 2 encapsulated strain kindly provided by Elaine Tuomanen (St. Jude Children's Research Hospital, Memphis, Tenn.), was grown in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) and frozen at −70°C in aliquots of known concentration.

Drug studies with mice. Animal studies with 8- to 10-week-old female 129x1/SvJ mice (weight, 18 to 20 g; Jackson Laboratories, Bar Harbor, Maine) were performed in a biosafety level 3 facility in the Animal Resource Center at St. Jude Children's Research Hospital. All experiments were done under general anesthesia with inhaled isoflurane 2.5% (Baxter Healthcare Corp., Deerfield, Ill.) and were approved by the Animal Care and Use Committee at St. Jude Children's Research Hospital.

Mice were infected by intranasal inoculation with 50 μl of sterile phosphate-buffered saline (PBS) containing a sublethal dose of $10^{5.5}$ $TCID_{50}$ of rSV(hHN) per mouse and challenged 7 days later with 100 μl of sterile PBS containing a sublethal dose of 25 CFU of *S. pneumoniae* per mouse. Treatment with BCX 2798 was by intranasal administration in a volume of 50 μl of sterile PBS twice daily for 5 days starting 4 h before or 24 h after virus exposure. BCX 2798 compound was administered to groups of mice ($n = 10$) at dosages of 0.1, 1, 10, and 50 mg/kg per day. Control (infected untreated) animals received PBS instead of drug. Administration of drug or PBS was done under light anesthesia with isoflurane, and care was taken to ensure that exposure to anesthesia was equal for all groups. Mice were observed daily for 21 days after the pneumococcal challenge for clinical signs of infection and for survival. The parameters for evaluation of antiviral activity of BCX 2798 included prevention of weight loss and death and prolongation of survival. Weight changes were calculated for each mouse as a percentage of its weight on day 0 before virus infection.

Lung viral and bacterial titers. Four mice from each group were sacrificed at 4, 48, and 96 h after pneumococcal infection for virus and bacterium titrations. Lungs were removed under sterile conditions, washed three times in PBS, and ground and suspended in a total volume of 1 ml. The suspensions for virus titration were centrifuged at 2,000 × *g* for 10 min to clear cellular debris. Virus titers were determined by plating tenfold dilutions of this suspension in a final volume of 1 ml on LLC-MK_2 cells in 24-well plates with minimal essential medium containing 0.1% bovine serum albumin in the presence of 1 μg of acetylated trypsin and 50 μg of gentamicin/ml to determine the $TCID_{50}$. Lung homogenates were used directly for bacterial cultures prior to centrifugation.

TABLE 1. Survival of 129x1/SvJ mice infected with rSV(hHN) or *S. pneumoniae*

Infectious agent and dose	No. of survivors/total no. tested	% Survival	MSD ± SD[a]
rSV(hHN) ($TCID_{50}$/mouse)			
10^5	15/15	100	ND
$10^{5.5}$	15/15	100	ND
10^6	12/15	80	8.5 ± 2.3
S. pneumoniae (CFU/mouse)			
25	10/10	100	ND
100	2/5	40	2.3 ± 0.6
400	0/5	0	2.8 ± 0.2

[a] MSD, mean survival day considering only mice that died; ND, not determined since no mice died.

TABLE 2. Effect of sequence of rSV(hHN) and *S. pneumoniae* inoculation on survival

Infectious agent(s)	No. of survivors/total no. tested	% Survival[a]	MSD ± SD[b]
Virus, then bacteria	0/8	0*	3.6 ± 0.5
PBS, then bacteria and virus	3/8	38*	4.3 ± 1.1
Bacteria, then virus	8/8	100	ND
Virus, then PBS	8/8	100	ND
PBS, then bacteria	8/8	100	ND

[a] *, $P < 0.05$ compared to singly infected groups.
[b] MSD, mean survival day considering only mice that died after the second challenge. ND, not determined since no mice died.

Quantitation of pneumococcal colony counts was done by using 10-fold dilutions on tryptic soy agar plates (Difco) supplemented with 3% (vol/vol) sheep erythrocytes.

Pathology. Lungs were removed on day 3 after the secondary challenge and fixed in 10% neutral buffered formalin for 24 h. Lungs were then embedded in paraffin, sectioned (5 μm), stained with hematoxylin and eosin, and examined microscopically for histopathologic changes by an experienced animal pathologist (K.L.B.) who was blinded to the composition of the study groups. The degree of involvement of lung sections was estimated by examination under low power for evidence of consolidation, whereas the type of involvement, including the specific cell types involved, was determined by examination under high power. The airways (bronchioles, alveolar ducts, etc.) were examined to see whether they contained inflammatory cells, epithelial necrosis, or epithelial hyperplasia.

Statistical analysis. Comparison of survival between groups of mice was done with the Mantel-Cox chi-square test on the Kaplan-Meier survival data. Comparison of viral and bacterial titers in lungs between groups was done with the Wilcoxon rank sum test. Comparisons of weight loss and mean day to death between groups were done by using the Student *t* test for pairwise comparisons and one-way analysis of variance, followed by Dunn's test for comparisons among multiple groups. The mean survival day was determined as the number of days surviving after infection considering only mice that died. A *P* value of <0.05 was considered significant for these comparisons.

RESULTS

Lethal synergism between rSV(hHN) and *S. pneumoniae*. The hPIVs are strict pathogens of humans and cause no disease in mice. To develop and characterize an animal model that can be used for evaluation of the potency of the BCX 2798 compound (HN inhibitor) against synergistic mortality between hPIV-1 and *S. pneumoniae*, we rescued rSV(hHN), in which the HN gene of SV was replaced with that of hPIV-1. Our previous data indicated that this recombinant virus was pathogenic for mice (1).

To establish that lethal synergism occurs between a paramyxovirus pseudotyped with the hPIV-1 HN and pneumococcus, we first determined the maximum infectious dose at which no mortality occurred for rSV(hHN) and for *S. pneumoniae* in 129x1/SvJ mice (Table 1). The sublethal doses of $10^{5.5}$ $TCID_{50}$ of recombinant virus per mouse and of 25 CFU of pneumococci per mouse caused moderate illness but no mortality of infected animals and were used for further experiments.

To determine the influence of the sequence between virus and bacterium inoculation on the level of synergistic mortality, we infected mice with the chosen sublethal doses of infectious agents or PBS (as a mock infection) 7 days apart in various combinations. All mice infected with recombinant virus and 7 days later challenged with *S. pneumoniae* died within a few days of pneumococcal challenge (Table 2). Fewer mice developed clinical illness and died in the group infected simultaneously with both infectious agents at day 7 after mock infection with PBS. No mortality was observed in mice infected with pneumococci and 7 days later challenged with recombinant virus, a finding which was similar to that for the control groups of mice infected with nonlethal doses of either rSV(hHN) or *S. pneumoniae* in conjunction with mock infection with PBS. These data indicate that lethal synergism between an SV pseudotyped with the hPIV-1 HN and pneumococcus can be demonstrated in mice. Since the administration of parainfluenza virus and then *S. pneumoniae* separately by 7 days caused the highest level of mortality of infected animals (compared to simultaneous or reverse administration), this scheme of delivery of infectious agents was used in our further experiments in the synergistic model.

To further characterize the model and determine the role of the virus dose in the development of a lethal viral-bacterial interaction, we infected mice with different sublethal doses of $10^{3.5}$, $10^{4.5}$, and $10^{5.5}$ $TCID_{50}$ of rSV(hHN) per mouse and challenged them 7 days later with *S. pneumoniae* at a sublethal dose of 25 CFU per mouse. Weight loss and synergistic mortality of mice from the secondary pneumococcal infection were dependent on the dose of inoculated virus (Fig. 2). Only one mouse out of 10 died in the group of animals infected with the lowest virus dose of $10^{3.5}$ $TCID_{50}$ and subsequently challenged with bacteria. Mice infected with the middle dose of $10^{4.5}$ $TCID_{50}$ of recombinant virus did not lose weight or show signs of infection before the pneumococcal challenge. Pneumococcal superinfection resulted in a modest (7% mean) weight loss by day 5 after secondary infection but led to mortality in 60% of the mice. Mice infected with the highest nonlethal virus dose of $10^{5.5}$ $TCID_{50}$ lost an average of 15% of their initial weight in the 7 days after the viral infection prior to bacterial challenge at day 0 and continued to lose weight after the secondary challenge with *S. pneumoniae*, dying by day 6. Thus, rSV(hHN) could predispose to lethal synergism with *S. pneumoniae* at doses lower than those needed to cause clinical symptoms or weight loss in singly infected animals. We next sought to utilize this mouse model of secondary bacterial pneumonia after rSV(hHN) infection as a tool for the evaluation of the potential for antiviral compounds to prevent bacterial complications of parainfluenza virus infections.

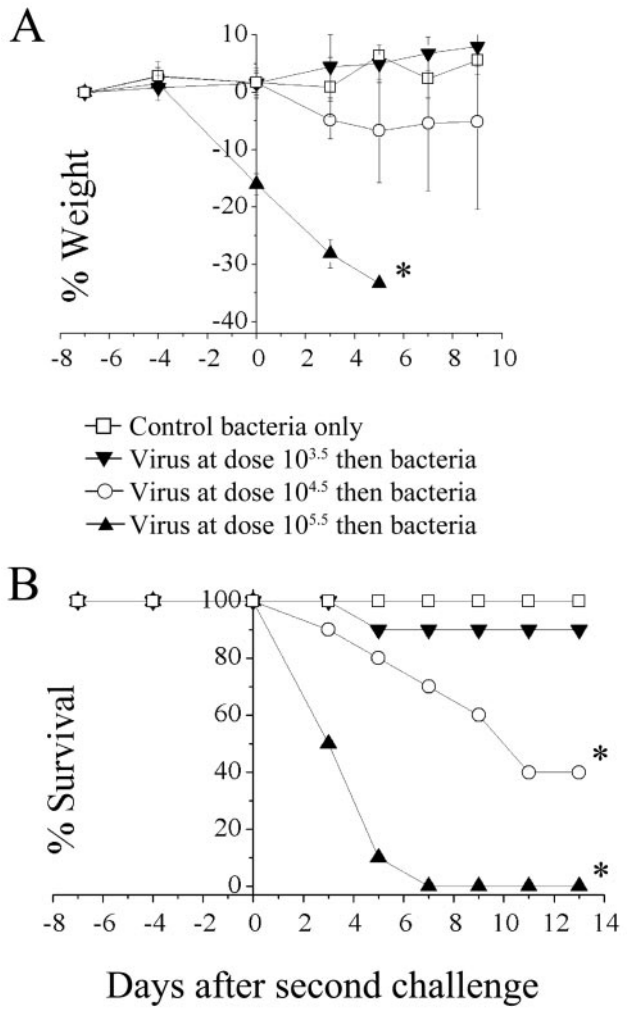

FIG. 2. Effect of different doses of rSV(hHN) on weight loss (A) and survival (B) of mice in a secondary pneumonia model. Mice ($n = 10$ per group) were infected with a sublethal dose of $10^{3.5}$, $10^{4.5}$, or $10^{5.5}$ $TCID_{50}$ per mouse of rSV(hHN) and then challenged 7 days later with a sublethal dose of 25 CFU of *S. pneumoniae* per mouse. Mice in a control virus group were challenged with PBS instead of bacteria and had 100% of survival (data not shown). Weight loss and survival of mice were monitored through 21 days after the bacterial challenge. Mean values for weight loss are plotted with error bars indicating the standard deviation. An asterisk indicates a significant difference in weight change or survival compared to the control groups infected with virus or bacteria alone ($P < 0.05$).

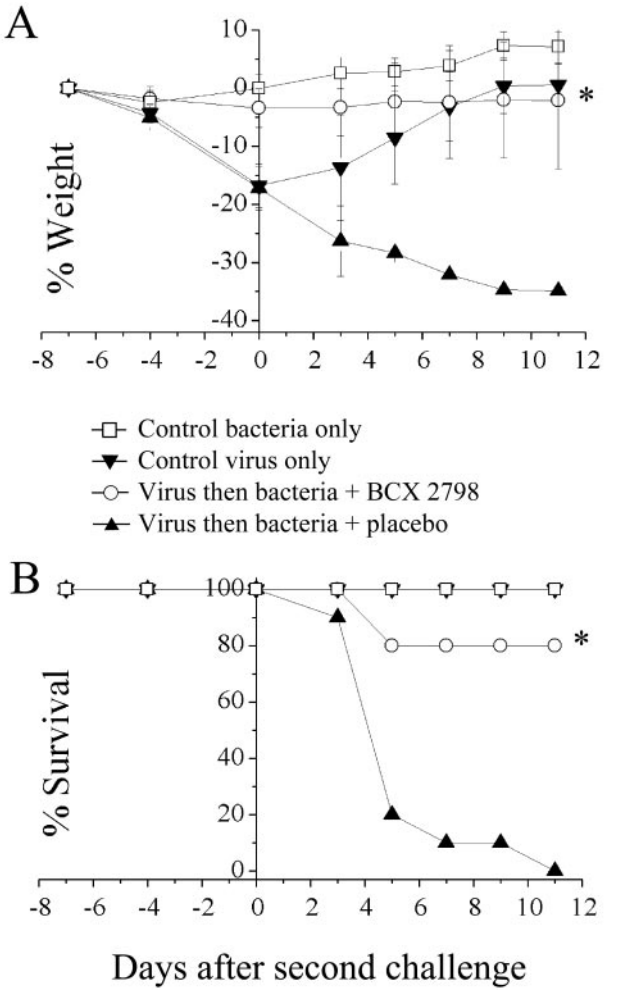

FIG. 3. Effect of BCX 2798 on weight loss (A) and survival (B) of mice in a secondary bacterial pneumonia model after rSV(hHN) infection. Mice ($n = 10$ per group) were infected with a sublethal dose of $10^{5.5}$ $TCID_{50}$ of rSV(hHN) per mouse and then challenged 7 days later with a sublethal dose of 25 CFU of *S. pneumoniae* per mouse. Mice in the control groups were infected either with virus or with bacteria alone. Treatment at 10 mg/kg per day with BCX 2798 or PBS as a placebo was started 4 h before viral infection. Weight loss and survival of mice were monitored through 21 days after the bacterial challenge. Mean values for weight loss are plotted with error bars indicating the standard deviation. An asterisk indicates a significant difference in weight change or survival for the drug-treated group compared to the synergism group receiving placebo ($P < 0.05$).

Efficacy of BCX 2798 against lethal synergism between rSV(hHN) and *S. pneumoniae*. To evaluate the efficacy of the BCX 2798 compound against lethal synergism between rSV(hHN) and *S. pneumoniae*, we infected groups of 10 mice with a sublethal dose of $10^{5.5}$ $TCID_{50}$ of recombinant virus per mouse and 7 days later challenged them with a sublethal dose of 25 CFU of pneumococci per mouse. Our previous toxicity experiments did not determine any abnormalities in uninfected mice (in terms of weight change and survival) treated with BCX 2798 at a dosage as high as 50 mg/kg per day (1).

Prophylactic treatment with BCX 2798 was administered to mice at doses of 0.1, 1, and 10 mg/kg per day in two divided doses, starting 4 h before viral infection.

Weight loss and survival of infected treated animals were compared to weight loss and survival of infected untreated animals and were monitored for 21 days after the bacterial challenge (Fig. 3). Mice infected with a sublethal dose of rSV(hHN) and challenged 7 days later with a sublethal dose of *S. pneumoniae* (placebo-treated synergism group) lost an average of 35% of their initial weight, and all died by day 11 after the secondary pneumococcal infection. Mice infected in the same manner but treated with BCX 2798 at 10 mg/kg per day starting 4 h before viral infection (BCX 2798 treated synergism group) lost an average of 3% of their initial weight by day 7 after the viral infection but did not lose further weight upon bacterial challenge. BCX 2798 at 10 mg/kg per day protected

mice from mortality as well, significantly improving survival to 80% compared to 0% in the placebo-treated group. Pretreatment of mice with the lower doses of BCX 2798 of 0.1 and 1 mg/kg per day did not prevent weight loss of infected animals after the second bacterial challenge and had no effect on synergistic mortality (data not shown).

To evaluate the efficacy of the BCX 2798 compound against lethal synergism between rSV(hHN) and *S. pneumoniae* in a delayed treatment model, BCX 2978 was administered to groups of mice at a dose of either 10 or 50 mg/kg per day beginning 24 h after initiation of the viral infection. No efficacy was observed comparing either weight loss or mortality at even the highest tested concentration. All mice in the drug-treated synergistic group died showing no differences in mortality or time to death compared to the placebo treated group of animals. These data indicate that BCX 2798 can prevent death of mice from secondary bacterial infection in our mouse model only when treatment is started before the onset of the viral infection.

Lung viral and bacterial titers. To further evaluate the model of synergistic mortality and the effect of prophylactic treatment in the model, we repeated the experiment utilizing BCX 2798 administered to mice 4 h before viral infection at a dosage of 10 mg/kg per day and measured bacterial and viral titers in mouse lungs. Lung titers were assayed in groups of 4 mice at 4, 48, and 96 h after the secondary bacterial infection (Fig. 4). Virus was only detected in the lungs of mice at the 4 h time point after bacterial challenge (or day 7 after viral infection), a result consistent with our earlier data demonstrating the clearing of rSV(hHN) from mouse lungs at doses of less than 10^6 $TCID_{50}$ before day 9 after viral infection (1). The drug-treated synergistic group of mice had 4 h postchallenge median viral lung titers ca. 2 logs lower than did placebo-treated mice or mice infected with recombinant virus alone (Fig. 4A). Bacterial superinfection did not increase virus load in mouse lungs and did not delay viral clearance. Lung bacterial titers were noted to increase significantly (ca. 4 logs) between the 4- and 48-h time points in both synergistic groups of mice but not in the control mice infected with *S. pneumoniae* alone (Fig. 4B). Bacterial titers in this last group were undetectable at all time points. Although we observed an increase in bacterial titers in both synergistic groups (BCX 2798-treated and placebo-treated mice) the bacterial titers in the placebo-treated group were significantly higher (~1 log) compared to the BCX 2798-treated group of mice. No titers were done in the untreated synergistic group of mice at 96 h after secondary challenge since all of them died before that time point. Thus, preceding parainfluenza virus infection led to an increase in lung bacterial titers, and protection of mice from lethal synergism by BCX 2798 was mediated by a reduction in lung bacterial titers as a result of inhibition of rSV(hHN) growth in mouse lungs.

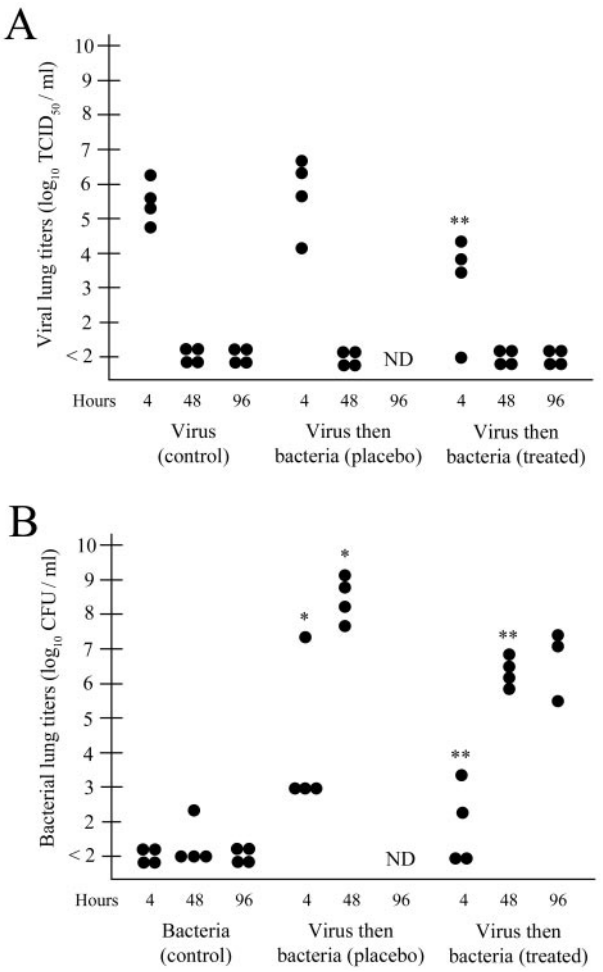

FIG. 4. Lung viral (A) and bacterial (B) titers in a secondary pneumonia model following rSV(hHN) infection. Mice ($n = 4$ per group) were infected with a sublethal dose of $10^{5.5}$ $TCID_{50}$ of rSV(hHN) per mouse and then challenged 7 days later with a sublethal dose of 25 CFU of *S. pneumoniae* per mouse. Mice in the control group were infected either with virus or bacteria alone. Treatment at 10 mg/kg per day with BCX 2798 or PBS was started 4 h before the viral infection in the treatment and placebo groups. Lungs were collected 4, 48, and 96 h after challenge with *S. pneumoniae*. Each point represents the results from a single mouse. A single asterisk indicates a significant difference in titers at that time point compared to the control group infected with a single agent ($P < 0.05$), and two asterisks indicate a significant difference in titers at that time point compared to the placebo group of mice. ND, not determined because of death of mice.

Histopathologic changes in the synergism model and the effect of BCX 2978. We next examined the lungs of mice in treated and untreated synergistic groups for histopathologic changes (Fig. 5). Groups of three mice were sequentially infected with combinations of PBS, rSV(hHN), and pneumococci as in earlier protection experiments utilizing BCX 2978, and lungs for histopathology were collected at day 3 after the second challenge. The mice in the PBS control group and the group infected with pneumococci after PBS were indistinguishable. Inflammation was not present in the examined lung sections (Fig. 5A and B), and bacteria could not be seen in animals infected with bacteria alone (Fig. 5B). Animals in the remaining three groups, however, had consistent and distinct histological changes. Animals infected with rSV(hHN) either followed by PBS (Fig. 5C) or bacteria (Fig. 5D) had characteristic changes involving the airways and interstitium. Alveolar spaces contained moderate infiltrates of lymphocytes, macrophages, and neutrophils and alveolar edema. In bronchi the mucosal epithelium was hyperplastic, with foci of mucosal necrosis and sloughing of degenerate cells into the bronchial lumen. Throughout the tissue there was prominent mononuclear cuffing of vessels. In addition to the described changes

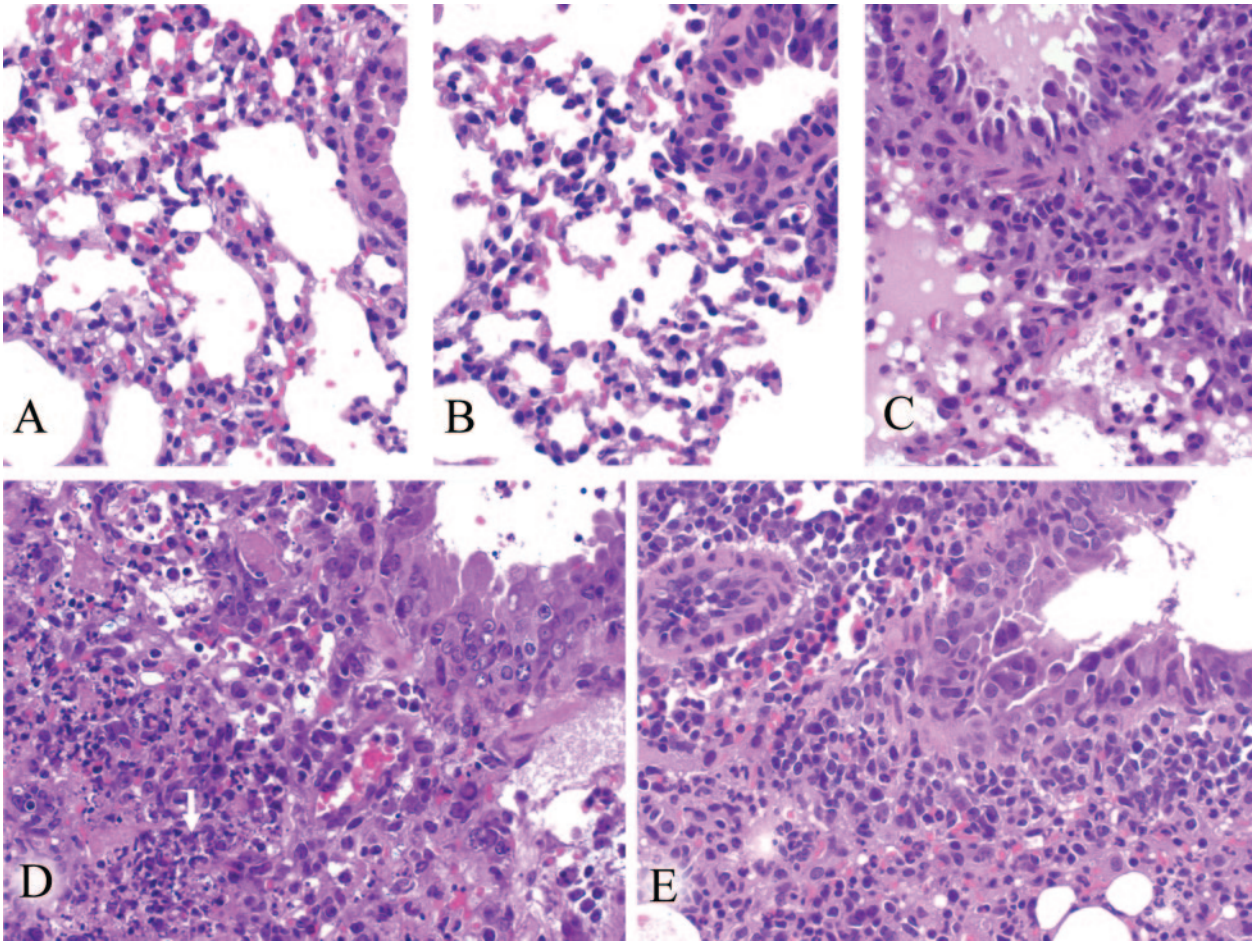

FIG. 5. Histopathologic changes in the lungs of mice infected in a secondary bacterial pneumonia model. Mice received PBS only (A); *S. pneumoniae* D39 at 25 CFU per mouse 7 days after PBS mock infection (B); rSV(hHN) at $10^{5.5}$ $TCID_{50}$ per mouse, followed 7 days later by PBS mock infection (C); rSV(hHN), followed 7 days later by pneumococci with placebo treatment (D); or rSV(hHN), followed 7 days later by pneumococci with BCX 2978 treatment (E). High-power magnification (×40) views of representative lung sections obtained 3 days after secondary challenge and stained with hematoxylin and eosin are pictured. The arrow in panel D highlights an area of extensive necrosis that is not present in the lungs of mice represented by panel E.

associated with viral infection alone, mice infected with rSV(hHN) and then pneumococcus had more severe consolidative and necrotic changes (Fig. 5D). In this group, two of three mice had extensive pneumonia resulting in consolidation of >80% of the lung tissue. In the severely affected areas, the alveolar walls were necrotic and the alveolar spaces were distended by plugs of fibrin, edema, and copious numbers of degenerate and viable neutrophils, macrophages, and lymphocytes. Airway lumens were filled by mucus, sloughed epithelium, and an exudate similar to that present in the alveoli. A mild fibrinopurulent pleuritis was present along the associated pleura. The third animal in this group had changes consistent with early infection, including inflammation, edema, and broncho-epithelial changes without parenchymal necrosis and fibrin deposition. In the group of mice given both virus and bacteria but treated with BCX 2978, the most obvious difference was in degree of involvement. Less than 50% of the lung tissue was involved, leading to a distinctly different appearance when whole lungs were examined (Fig. 5E). In addition, there was much less fibrin deposition and necrosis compared to the placebo group in the most severely affected areas of the parenchyma. Thus, BCX 2798 protected the lungs from progression to the widespread consolidation and necrosis seen during secondary bacterial pneumonia in this model.

DISCUSSION

Study of antimicrobial agents is facilitated by relevant small animal models of disease. A recently developed mouse model of influenza virus-pneumococcal synergism (32) has allowed testing of the efficacy of antiviral compounds for the treatment of resulting secondary bacterial infections (30, 31). However, experimental evidence supporting the role of hPIVs in secondary bacterial infections has been lacking since small animal models of hPIV infection that reproduce clinical disease in humans do not exist. In vivo studies with the closely related mouse parainfluenza virus 1 (SV) supported the concept that PIVs can prime for pneumonia from bacteria including *Haemophilus influenzae*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Mycoplasma pulmonis*, and *Pasteurella pneumotropica*

(10, 22, 23, 24). Therefore, we utilized a recently developed paramyxovirus mouse model with rSV(hHN) (1) that took advantage of the growth and disease-producing properties of SV. The induction of lethal synergy between this recombinant virus and *S. pneumoniae* allowed testing of the novel compound BCX-2978 in the system. We found that inhibition of the HN of hPIV-1 altered the course of secondary bacterial pneumonia and prevented death.

Our experiments demonstrate that the recombinant virus rSV(hHN) can prime mice for the development of pneumococcal pneumonia. To achieve synergistic killing, virus infection must precede the bacterial challenge since preinfection with pneumococci did not increase morbidity or mortality from rSV(hHN), and simultaneous administration caused only mild enhancement of disease. The magnitude of the effect on mortality was related to the dose of virus administered, although doses below those needed to cause clinical symptoms in mice could prime for lethal infection. Thus, in some respects the model is similar to that developed for influenza virus and pneumococcus (32), reinforcing the concept that respiratory viruses other than influenza virus predispose to secondary bacterial infections.

The novel parainfluenza virus inhibitor BCX 2978 is a rationally designed compound based on the crystal structure of the HN of NDV. The drug binds the active site of the HN of hPIVs, inhibiting both hemagglutination and NA activity over a broad range of concentrations (1). Because both binding and NA activities are contained in a single site, inhibition of the hPIV-1 HN by competitive blockade likely has different effects on the virus life cycle than inhibitors of the influenza virus NA have on influenza virus. In earlier experiments, we demonstrated that inhibition of the influenza virus NA by prophylactic treatment with oseltamivir decreased viral replication and inhibited lethal synergism with pneumococcus in a mouse model, similar to the results we report here (29). This effect was specific to the NA and was thought to be mediated by cleavage of sialic acids by the viral NA during replication and spread of the virus throughout the respiratory tract, exposing receptors for pneumococcal adherence and invasion. However, a separate treatment effect was seen in the influenza virus model that was dependent on inhibition of NA activity but was independent of viral replication, implying other functions for the influenza virus NA in the context of a secondary infection. This effect of delayed treatment was not seen in the current study using rSV(hHN). The reasons for this are unclear. A reduced dependency on NA activity for viral spread is one potential explanation, since the hPIV-1 HN has relatively low NA activity compared to SV or influenza viruses. The ability of BCX 2978 to inhibit synergism when viral replication is decreased but not in a delayed treatment model when viral replication remains unchanged indicates that the contribution of the viral NA is less in this model than in the previous model utilizing influenza virus. The similar spectrum of synergistic interaction shared between the hPIVs and RSV, a virus that lacks NA activity, supports this argument. Since the HN switches between conformations suitable for binding or for hydrolysis, both of which take place at the same site (8, 9), NA activity may not be expressed at the time it would be needed for support of bacterial adherence. Thus, inhibition of the HN would have no effect on this proposed mechanism of synergism.

It is more likely that inhibition of viral spread through blockade of attachment at the HN site, or a combined effect on attachment and NA activity, is responsible for the reduction in secondary bacterial disease. The main effect seen in mice superinfected with pneumococci was an increase in the lung bacterial load. This increase was rapid over the first 48 h and resulted in severe consolidation from an inflammatory infiltrate throughout the lungs. The degree of consolidation throughout the lungs and the presence of necrosis were striking differences from the characteristic pneumococcal pneumonia seen with bacterial infection alone. Interestingly, the virus titers of rSV(hHN) were not increased in pneumococcal superinfected mice. This failure to augment viral replication is another difference compared to the influenza model (30) that may provide clues as to the mechanism. In the prior model, bacterial infection increased the lung influenza virus load by >10-fold, and prophylactic drug treatment prevented this bacterium-mediated increase (29). Although pneumococcus did not increase lung titers of rSV(hHN) in the absence of drug in the present study, treatment with BCX 2978 led to lower lung titers of both bacteria and virus. This resulted in a change in the distribution of lesions in the lungs but not in the character of the lesions. Necrosis and inflammatory infiltrates were present, but the pathological changes were limited in extent and more closely resembled early stages of infection. Thus, the effect of viral inhibition was to slow the progression of secondary bacterial pneumonia that likely allowed the immune system to clear the infection before it overwhelmed the animals. Although much recent attention has been focused on mechanisms by which paramyxoviruses suppress interferons (15, 27), it is not clear how this might benefit bacteria. It is not known whether these viruses have other effects on the innate and adaptive immune systems that might enhance bacterial growth.

The interaction between paramyxoviruses and bacteria is clinically significant. The hPIVs have a widespread distribution in the world and are one of the leading causes of hospitalization from respiratory virus infections in children (40). The hPIVs have a role in the development of bacterial otitis media and secondary bacterial pneumonia, and the present study underscores the importance of this interaction. Development of secondary bacterial complications as a result of primary PIV disease increases the economic impact of viral infection enormously. Our results suggest that an approach using selective inhibitors of the HN of PIVs might be reasonable not only for the amelioration of viral disease but also for prevention of secondary bacterial complications. Although our data in a mouse model support such a role for prophylaxis but not treatment, use of antiviral agents active against paramyxoviruses for this purpose should be studied in humans in both clinical settings. Intervention would be expected to be especially efficacious in children, the elderly population, immunosuppressed persons, and in patients with antibiotic treatment failure. These findings support further drug development and highlight the need for clinical trials with humans to address these issues.

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